Effects of Actin Filament Cross-linking and Filament Length on Actin–Myosin Interaction

THOMAS R. COLEMAN and MARK S. MOOSEKER
Department of Biology, Yale University, New Haven, Connecticut 06511

ABSTRACT We have used two actin-binding proteins of the intestinal brush border, TW 260/240 and villin, to examine the effects of filament cross-linking and filament length on myosin–actin interactions. TW 260/240 is a nonerythroid spectrin that is a potent cross-linker of actin filaments. In the presence of this cross-linker we observed a concentration-dependent enhancement of skeletal muscle actomyosin ATPase activity (150–560% of control; maximum enhancement at a 1:70–80 TW 260/240:actin molar ratio). TW 260/240 did not cause a similar enhancement of either acto-heavy meromyosin (HMM) ATPase or acto-myosin subfragment-one ($S_1$) ATPase.

Villin, a Ca$^{2+}$-dependent filament capping and severing protein of the intestinal microvillus, was used to generate populations of actin filaments of various lengths from <20 nm to 2.0 μm; (villin:actin ratios of 1:2 to 1:4,000). The effect of filament length on actomyosin ATPase was biphasic. At villin:actin molar ratios of 1:2–25 actin-activated myosin ATPase activity was inhibited to 20–80% of control values, with maximum inhibition observed at the highest villin:actin ratio. The ATPase activities of acto-HMM and acto-$S_1$ were also inhibited at these short filament lengths. At intermediate filament lengths generated at villin:actin ratios of 1:40–400 (average lengths 0.26–1.1 μm) an enhancement of actomyosin ATPase was observed (130–260% of controls), with a maximum enhancement at average filament lengths of 0.5 μm. The levels of actomyosin ATPase fell off to control values at low concentrations of villin where filament length distributions were almost those of controls. Unlike intact myosin, the actin-activated ATPase of neither HMM nor $S_1$ showed an enhancement at these intermediate actin filament lengths.

In the cell, myosin interacts with actin filaments of defined length which are often tethered to other structures such as the plasma membrane or other actin filaments. Many specific actin-binding proteins have been identified which could function in vivo to regulate either actin filament length or the cross-linking state of filaments (for reviews see references 14 and 32). Thus, to understand how actin–myosin interactions generate movement in vivo, it is necessary to consider not only the isolated interaction of actin filaments with myosin but also the possible effect of these various classes of actin binding proteins on this interaction.

In the present study, we have used two well-characterized actin-binding proteins to examine the effect of both actin filament cross-linking and actin filament length on the ATPase activity of myosin. For the cross-linking study, we used TW 260/240, a tissue-specific spectrin of the avian intestinal brush border (11), which has been shown to be a potent cross-linker of actin filaments (9, 20). This particular actin filament cross-linker was selected based on preliminary evidence of Shimo-Oka and Watanabe (23) that a crude preparation of fodrin, a nonerythroid spectrin from mammalian brain, stimulated actomyosin ATPase. These preliminary results were confirmed and extended by Wagner (29) while our own studies were in progress. To test the effect of filament length on the actin–myosin interaction, we used various concentrations of the Ca$^{2+}$-dependent actin capping and severing protein, villin, to generate populations of actin filaments of various lengths (for reviews see references 6 and 17). The results presented here have appeared in preliminary form elsewhere (4).

MATERIALS AND METHODS

Preparation of the Brush Borders: Brush borders were isolated from the small intestines of chickens according to the method of Mooseker et al.
Published November 1, 1985

Isolation of TW 260/240, Villin, Actin, Myosin, HMM, and S1: TW 260/240 was purified by a modification of the methods of Glenney et al. (11) as described by Pearl et al. (20). Fractions of purified TW 260/240 from the gel filtration column were concentrated by dialysis against the brush border stabilization solution containing ammonium sulfate to 65% saturation. The precipitated protein was brought up in a minimal volume of 150 mM KCl, 10 mM imidazole-Cl, pH 7.2, 1.0 mM MgCl2, 0.02% NaN3, 0.2 mM dithiothreitol (DTT), with 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 20 TIU/liter aprotinin.

Villin was isolated by a modification of the method of Mooseker et al. (19). In brief, villin-containing fractions from the above gel filtration column were pooled and dialyzed extensively against several changes of column buffer (20 mM KCl, 0.2 mM CaCl2, 10 mM Tris-Cl, pH 8.2, 0.2 mM DTT, 0.2 mM PMSF, 20 TIU/liter aprotinin). Villin was purified on a 10-ml DEAE A-25 column (Sigma Chemical Co.) using a sharp linear KCl gradient (20 to 600 mM; 25 ml per side of chamber) in the above column buffer. After purification, villin was dialyzed against 75 mM KCl, 10 mM imidazole, pH 7.2, 1.0 mM MgCl2, 0.02% NaN3, 0.2 mM DTT with 0.2 mM PMSF and 20 TIU/liter aprotinin and was used within 2 wk of purification.

Chicken breast muscle actin was prepared from acetone powders following the method of Spudich and Watt (26).

Chicken skeletal muscle myosin was prepared following the method of either Margossian and Lowey (15) or Kielley and Harrington (13) with the substitution of 20 mM imidazole-Cl (pH 7.0) for potassium phosphate buffer in the extraction solution.

Both HMM and S1 were separated from intact myosin and tail fragments following the methods of Weeds and Taylor (34) with 20 mM imidazole-Cl substituted for the suggested sodium phosphate. HMM and S1 were separated from intact myosin by size exclusion chromatography on a high performance liquid chromatography column (Biogel A 5.0, 200-400 mesh; Bio-Rad Laboratories, Richmond, CA) followed by purification with either concentrated stocks that contained trace amounts of 32P-3',5'-ATP. Protein concentrations used were: TW 260/240, 12.0 cm-1 M; villin, 13.0 cm-1 M; actin, 13.0 cm-1 M. Molar ratios of actin:TW 260/240 were determined assuming a dimer M of 500,000.

ATPase Assays: Two ATPase assays were used. The first was based on Pollard and Korn's (21) modification of Martin and Doty's (16) procedure and the second was Taussky and Schorr's (27) colorimetric method. Both assays were performed at 30°C in a final assay solution of 66 mM KCI, 5 mM MgCl2, 0.2 mM CaCl2 or 0.5 mM EGTA, 8 or 20 mM imidazole, pH 7.2, and 2 mM DTT. ATPase assays were performed (at the suggestion of one of the reviewers) because no attempts were made to determine how much ATP remained in the acto-HMM assay samples were diluted 1:5 in assay buffer without ATP. In some experiments (see Fig. 7) the assay mixtures were stored on ice overnight before staining. No attempt was made to determine how much ATP remained in the assay mixtures at the time of staining. Lengths of villin-actin filaments were measured directly from enlarged micrographs using an electronic planimeter (Numonics model 1224 Electronic Digitizer, Numonics Corp., Lansdale, PA). The shortest filaments that could be measured were at 1:8 molar ratio. At higher ratios (>1:2-1:5) the villin-actin filaments were visualized as globular oligomeric complexes that generally had diameters <20 nm.

RESULTS

Effect of Actin Filament Cross-linking on Actomyosin, -HMM, and -S1 ATPases

We examined the concentration dependence of the effect of TW 260/240 on actin-activated myosin Mg2+-ATPase. Under the conditions used we usually obtained a 7- to 30-fold increase in myosin ATPase in the presence of actin alone. In the presence of TW 260/240 we observed an enhancement of actomyosin ATPase at ratios as low as 1:300. This potentiation of activity reached a maximum in the range of 1:80 (Fig. 1). Although the extent of this enhancement varied with the TW 260/240 preparation (150% to 560%), the peak activation was invariably at about the same ratio of TW 260/240:actin (1:70 to 1:80). Higher ratios of TW 260/240:actin (>1:40) were not routinely examined since the macroscopic aggregates of "gelled" actin filaments that are formed effectively remove actin from solution (see reference 20). Actomyosin ATPase assays at these higher concentrations of TW 260/240 show significant but highly variable inhibition of actin activation (data not shown). Similar results have been reported by Dabrowska et al. (8) for the effect of high cross-linking concentrations of fimbrin on actomyosin ATPase, and on acto-HMM ATPase by Davies et al. (7). Control preparations, including TW 260/240 alone, TW 260/240 plus actin, and TW 260/240 plus myosin, had little Mg2+-ATPase activity (results not shown).

While the present study was in progress, Wagner (29) published a paper reporting on the effect of fodrin purified from bovine brain on actomyosin ATPase. Similar to the results of the present study, results from that study showed that fodrin also has a "super-activating" effect on the actin-activated myosin ATPase. Furthermore, the addition of fodrin caused the actomyosin ATPase to be Ca2+-sensitive. In the presence of Ca2+, fodrin-stimulated actomyosin ATPase, whereas in the presence of EGTA fodrin inhibited actomyosin ATPase (29). Unlike the situation with fodrin, we observed no significant Ca2+-dependence on the enhancement of actomyosin ATPase by TW 260/240 (Fig. 2). The range of enhancement with or without Ca2+ in various preparations of actomyosin in the presence of TW 260/240 (at a 1:70 molar ratio) was 340-370% of control values (Fig. 2).

The effect of TW 260/240 on acto-S1 and -HMM ATPases was also examined. Two different ionic strength assay conditions were examined, assay buffer containing 66 mM added KCl (the same conditions used for the above actomyosin assays) and 13 mM KCl. The lower ionic strength assays were conducted (at the suggestion of one of the reviewers) because both S1 and HMM exhibit much higher actin-activated ATPase activities under these conditions. At this low ionic strength the actin-activated ATPase of intact myosin still showed a significant enhancement above control levels in the

---

1 Abbreviations used in this paper: C, critical concentration; DTT, dithiothreitol; HMM, heavy meromyosin; PMSF, phenylmethylsulfonyl fluoride; S1, myosin subfragment-one; TIU, trypsin inhibitor unit.
Figure 1 Concentration dependence of TW 260/240 on actomyosin (●, ○), acto-S₁ (▲, △), and acto-HMM (●, ○) ATPases. Buffer conditions were 5 mM MgCl₂, 0.2 mM CaCl₂ with either 20 mM imidazole, pH 7.2 (●, ○, ▲, △), or 8 mM imidazole, pH 7.2 (●), and either 66 mM KCl (●, ▲, ○) or 13 mM KCl (●, △, ○). The control levels of ATPase in the absence of TW 260/240 were: myosin, 0.025 (●), 0.077 (○); actomyosin, 0.174 (●), 0.283 (○); S₁, 0.106 (▲), 0.083 (△); acto-S₁, 0.303 (▲), 1.647 (△); HMM, 0.060 (●), 0.057 (○); acto-HMM, 0.364 (●), 1.060 (○) where units are micromolar inorganic phosphorus (Pᵢ) per milligram enzyme per minute. Graphs are plotted as percent activity of the actomyosin, acto-S₁, or acto-HMM control preparations. The molar ratio of TW 260/240:actin is also expressed on the abscissa.

Figure 2 Effect of Ca²⁺ on the enhancement of actomyosin ATPase in the presence of TW 260/240. Assay conditions were as in Fig. 1 with either 0.1 mM CaCl₂ or 0.5 mM EGTA and a TW 260/240:actin molar ratio of 1:70. AM, actomyosin.

Effect of Filament Length on Actomyosin, -HMM, and -S₁ ATPases

The effect of filament length on actomyosin ATPase was examined using various concentrations of the Ca²⁺-dependent capping and severing protein, villin, to generate populations of actin filaments of varied lengths. As shown by electron microscopy (5) and viscometry (19), the length of actin filaments achieved at steady state is, in a qualitative sense, inversely proportional to the concentration of villin present. Thus we were able to generate filament lengths from <20 nm (at a 1:2 molar ratio) to ~2 µm by merely varying the concentration of villin (Figs. 4 and 5, and Table I). Control filament preparations in the absence of villin attained average lengths between 2 and 3 µm.

Myosin ATPase assays were performed using “control” actin and villin-actin preparations. At villin:actin molar ratios between 1:2 and 1:25 (average lengths <20–200 nm) actomyosin ATPase activity was inhibited by 20–80%, the extent of inhibition increasing with villin concentration (Fig. 6). One possible reason the lack of activation of myosin ATPase by short villin-actin filaments that could be examined directly was that short filaments formed by nucleated assembly in the presence of villin contain primarily ATP-actin monomers, since it has recently been shown that hydrolysis of bound ATP lags behind monomer addition (for review see reference 35). To test this possibility, we prepared short filaments by adding villin to preassembled F-actin. Such severed filaments should contain predominantly ADP monomers. We observed no difference in the inhibition of myosin ATPase by short villin-actin filaments that could be examined directly was that short filaments formed by nucleated assembly in the presence of villin contain primarily ATP-actin monomers, since it has recently been shown that hydrolysis of bound ATP lags behind monomer addition (for review see reference 35). To test this possibility, we prepared short filaments by adding villin to preassembled F-actin. Such severed filaments should contain predominantly ADP monomers. We observed no difference in the inhibition of actomyosin ATPase by such short filaments generated by either severing or co-assembly with villin. For example, in one experiment at a 1:5 villin:actin ratio, both short filament populations activated myosin ATPase to only 35% of control actomyosin.

At intermediate actin filament lengths (average lengths 0.26–1.1 µm) generated at ratios of 1:40–1:400, enhancement of actomyosin ATPase was observed (130–260% of controls; maximum activation at 1:70–400). The extent of enhanced activation then fell off to control values at low concentrations of villin (1:2,000–4,000), where filament length distributions were nearly at control levels (Fig. 5, Table I). Control prepap-
rations, including villin alone, villin plus actin, and villin plus myosin, had little Mg\textsuperscript{2+}-ATPase activity (data not shown). On the other hand, the shearing of control actin preparations immediately before the assay was started (by vigorous, repeated pipetting), which produced shorter filaments (average length 0.98 μm) than unsheared controls (average length 2.62 μm), also enhanced myosin ATPase, with ATPase levels ~200% of control samples.

Like myosin, both acto-HMM and acto-S1 ATPase activities were inhibited at high ratios of villin:actin (Fig. 6). At intermediate actin filament lengths, however, neither acto-HMM nor acto-S1 showed the enhancement of actin-activated ATPase observed using intact myosin (Fig. 6). These two results—the inhibition with short filaments and the lack of enhancement with intermediate lengths—occurred at both 66 and 7 mM KCl (Fig. 6).

As in the TW 260/240 cross-linking experiment above, ultrastructural examination of these preparations revealed the lack of any myosin thick filaments (Fig. 4). In several experiments, however, the assay mixtures were incubated overnight before negative staining. Under these conditions, myosin thick filaments were observed (Fig. 7), but only in those mixtures that contained very short filaments generated at high villin:actin ratios (1:2–10).

DISCUSSION

The Effect of TW 260/240 on Actin–Myosin Interaction

The results presented here demonstrate that the tissue-specific spectrin of the avian brush border, TW 260/240, enhances the actin-activated Mg\textsuperscript{2+}-ATPase activity of skeletal muscle myosin. We attribute this effect to the cross-linking of actin filaments by TW 260/240 rather than a direct interaction of TW 260/240 and myosin. We have, in fact, been unable to demonstrate any direct binding interactions between TW 260/240 and myosin. For example, TW 260/240 does not co-assemble with or inhibit the formation of myosin thick filaments (data not shown). Furthermore, the cross-linking notion is supported by the observation that other proteins that cross-link actin filaments—such as alpha-actinin (3, 24), filamin (8, 25), and fodrin, a nonerythroid spectrin purified from mammalian brain tissue (22, 23, 29)—also enhance myosin ATPase activity over that observed in the absence of an actin cross-linker. See, for example, Fig. 6 of reference 8, which depicts the concentration dependence of filamin on actomyosin ATPase, demonstrating effects similar to those in Fig. 1 of this paper. In addition, like fodrin (29) and filamin (8), which do not enhance acto-S1 ATPase, the enhancement of myosin ATPase by TW 260/240 is observed only with intact myosin, not with either S1 or HMM.

Assuming that it is the cross-linking of actin filaments by TW 260/240 that results in the enhancement of acto-myosin ATPase, there are a number of possible explanations for such an effect. The simplest is that cross-linking of actin filaments increased the local concentration of actin binding sites accessible to a given myosin head. However, this mechanism fails to explain why no enhancement is observed when either S1 or HMM rather than myosin is used (Fig. 1). Another possibility is raised by the fact that cross-linking of filaments presumably will allow production of tension by myosin. Perhaps myosin works better under tension, but we are unaware of any evidence for this speculation. A problem with this idea is that myosin thick filaments, which might be necessary for force production, are not observed in the assay mixtures we have used in these experiments (Fig. 3). However, smaller...
bipolar arrays of myosin could provide tension and be unnoticed due to the resolution of negative stain.

**Effect of Filament Length on Actin–Myosin Interaction**

The underlying assumption in the studies presented here on the effect of filament length on actin–myosin interaction is that all of the villin–actin mixtures used are identical except for differences in filament length and number-concentration of filaments present. Previous studies have shown that as a result of barbed-end capping (2, 10, 17) villin causes a shift in the critical concentration ($C_c$) of actin to that of the pointed end (1). Under the conditions of this assay the $C_c$ is increased from ~0.1 to ~0.6 μM (1, 30, 31). This shift in $C_c$ is saturated at very low villin:actin ratios (30, 31), such that except for the very low concentrations of villin used (1:4,000), all of the villin mixtures should have the same polymer concentration, which, in fact, is slightly lower than the total polymer concentration of the control actin in the absence of villin. Since villin–actin mixtures of 1:40–400 actually yield higher levels of actomyosin ATPase, this slight villin-dependent decrease...
in total polymer clearly has no significant effect on these assays. One caution, however, is that the two studies (30, 31) that have examined the effect of villin on the C₀ used villin concentrations over a molar ratio range of 1:10–1,000. Thus the actual C₀ at very high villin ratios has not yet been determined directly and conceivably could be different from that measured in these initial studies. For example, if the C₀ for very short filaments were much greater than C₀'s previously observed, inhibition could simply be due to the presence of substantially less actin polymer. However, electron microscopy of all villin–actin mixtures, even at very high villin:actin ratios, revealed ample amounts of polymer present (Fig. 4). Another caution is that villin may in some way alter the actual structure of F-actin (see below).

Keeping in mind the above reservations, it is possible to consider several mechanisms for the biphasic effect of filament length on actin-myosin ATPase. The enhancement of actin-activated myosin ATPase by filaments of intermediate length (0.26 to 1.1 μm) indicates that in vitro at least, there is an optimum length of filament for interaction with myosin. Since a similar effect is not observed using either HMM or S₁, it seems likely that the increased number-concentration of filaments present at intermediate filament lengths as compared to control preparations is not the main causal factor in this enhancement. One possible explanation is that the intact myosin molecule is in an organizational state (albeit not in

![Image](https://example.com/image1.png)

**Figure 5** Effect of villin on actin filament length. Average filament lengths as a function of villin concentration for the two villin preparations (■, ○) used in the ATPase assays of Fig. 5. The molar ratio of villin:actin is also indicated on the abscissa. Control filament preparations in the absence of villin attained average lengths between 2 and 3 μm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Filaments counted</th>
<th>Average length</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>No villin</td>
<td>228</td>
<td>2.14</td>
<td>1.79</td>
</tr>
<tr>
<td>1:4,000</td>
<td>113</td>
<td>2.00</td>
<td>1.38</td>
</tr>
<tr>
<td>1:2,000</td>
<td>262</td>
<td>1.89</td>
<td>1.57</td>
</tr>
<tr>
<td>1:700</td>
<td>231</td>
<td>1.39</td>
<td>1.08</td>
</tr>
<tr>
<td>1:150</td>
<td>234</td>
<td>0.50</td>
<td>0.44</td>
</tr>
<tr>
<td>1:30</td>
<td>222</td>
<td>0.29</td>
<td>0.28</td>
</tr>
<tr>
<td>1:8</td>
<td>335</td>
<td>0.16</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>292</td>
<td>0.08</td>
<td>0.08</td>
</tr>
</tbody>
</table>

![Image](https://example.com/image2.png)

**Figure 6** Concentration dependence of villin on actomyosin (■, ○), acto-S₁ (▲, △), and acto-HMM (○) ATPase. Buffer conditions were as in Fig. 1 with either 8 mM imidazole (■, ○, △) or 20 mM imidazole (▲, △) and either 66 mM KCl (■, ○, △) or 7 mM KCl (▲, △). The control levels of ATPase in the absence of villin were: myosin, 0.030 (■), 0.026 (○); actomyosin, 0.192 (■), 0.320 (○); S₁, 0.080 (▲), 0.137 (△); acto-S₁, 0.450 (▲), 1.560 (△); HMM, 0.084 (○); acto-HMM, 1.618 (○) where units are micromolar inorganic phosphorus per milligram enzyme per minute. Graphs are plotted as percent activity of the actomyosin, acto-S₁, or acto-HMM control preparations. The molar ratio of villin:actin is also expressed on the abscissa.
The myosin molecule. That the potentiation of actomyosin ATPase by filaments of intermediate lengths is strictly an immediate length, perhaps as a result of more efficient packing of these shorter-than-control filaments around the heads of the myosin molecule. That the potentiation of actomyosin ATPase by filaments of intermediate lengths is strictly an effect of length and not some effect of villin on those filaments is strongly supported by the observation that similar enhancement is seen with control actin filaments vigorously sheared immediately before use in the ATPase assay.

The inhibition of the activities of both actomyosin and acto-S, by very short filaments generated at high villin:actin ratios (1:2 to 1:25) suggests that there might be some minimum filament length necessary for efficient myosin-actin interaction. Perhaps myosin molecules “travel” down a single filament for multiple cycles of ATP hydrolysis, just as myosin might do in muscle contraction. In this case, interactions with a short filament would be less efficient, since the myosin would fall off a given filament more frequently. Alternatively, short villin-actin filaments might be structurally different from control filaments, resulting in a lower affinity for myosin. Villin bound to the barbed end of very short filaments might directly hinder myosin-actin binding through steric interference. A more interesting possibility is that villin alters the conformation of monomers within these short filaments, an effect that can be propagated only for a short distance down the polymer from the villin-capped barbed end. Although deciphering the molecular basis for the ineffectiveness of short villin-actin filaments to activate myosin ATPase will require further experiments, it is clear that the results presented here in these initial studies establish yet another way in which filament-severing proteins such as villin might regulate not only cytoplasmic structure but also contractility in the cell.

We thank Tom Keller, Karen Conzelman, and Ed Edelman for insightful discussion during the course of this work. We also acknowledge the excellent technical support of Anne Goglia, Michael Boyd, and John Santarelli, and the assistance of Kris Mooseker in manuscript preparation.

This material is based upon work supported under a National Science Foundation Graduate Fellowship to T. Coleman and by both March of Dimes Grant 1-924 and National Institutes of Health grant AM 25387 to M. Mooseker.

Received for publication 8 May 1985, and in revised form 5 August 1985.

REFERENCES


